

ATP-BINDING APTAMER-INCORPORATED DNA HYDROGEL

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by

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ABSTRACT

Adenosine triphosphate (ATP) is a universal source of energy for all living organisms. It plays a fundamental intracellular and extracellular role in regulation of cellular metabolism, cell signal pathways and biochemical reaction. Its fundamental research and diverging application developed really fast, especially after the discovery of ATP aptamer. With a great development, the ATP aptamer has been applied to a wide range of area, such as biosensor, biomarker and some other detection. In view of the flexibility and versatile sequence and structure of nucleic acid, aptamer technology can be used at almost everywhere and its advantage is also striking, in lots of aspects even better than the antibody. At the same time, rolling circle amplification is in great booming, which can be utilized to rapidly copy multiple of DNA sequence by a circular DNA. Its repeated nucleic acid sequence, provide a really good vector for ATP aptamer in application. In my experiment, we produce a RCA-dependent ATP aptamer-incorporated DNA hydrogel to measure the amount of ATP that can be captured by DNA hydrogel.

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It's a great honor to be a graduate student in Cornell University and I really appreciate that I can get the chances to meet the unbeatable professors, friends and coworkers.

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CHAPTER 1

INTRODUCTION

1.1 Problem Statement

Adenosine triphosphate (ATP) is a universal source of energy for all living organisms. It plays a fundamental intracellular role in regulation of cellular metabolism and cell signal pathways inside the cell. It has been used as an indicator for cell development and cell injury (Wang, 2005). In addition, it is also an important extracellular messenger in neural tissues (Brake, 1994). It can bind and activate several cell-surface receptors, such as ion channels receptor, G-protein-coupled receptors to control many sorts of cellular biochemical reactions respectively (Burnstock, 1990). In view of its significant role inside cell, it attracted intense interest. Investigators started to use different kinds of methods to get various aspects of information on ATP. Cross, R. L figured out the variation on the mechanism of ATP formation by using FI-ATPases (Cross, 1981), which leads the straight study into the ATP hydrolysis reaction. A few years later, two groups of researchers (Ellington, 1990) used their in-vitro selection and amplification to successfully isolate certain RNA sequences that could specifically bind to some target molecules, from pools of random-sequence libraries (Tuerk, 1990). These kinds of sequence were termed RNA-binding aptamer. Later, the DNA-binding aptamer were also found (Ellington, 1992). With further study, researchers found that DNA/RNA aptamers can be identified as binding to a

wide range of targets, and ATP is one of them (Sassanfar, 1993). What's more, a part of aptamers possess high affinity for targets, which due to their flexible mechanism of folding in the process. Since the discovery of aptamer-based technology, nucleic acid aptamers have frequently been used in biosensor (Navani, 2006 & Cho, 2005), detection (Zheng, 2009) and new idea development in pharmacology (Chou, 2005). This gives investigators a great inspiration and possibility to master ATP. By polymerase chain reaction (PCR) or quantitative polymerase chain reaction amplification, an APT-aptamer-based DNA solution can be used to capture and transfer non-quantitative or quantitative ATP, but the sensitivity of this method so far doesn't meet the requirement for further development.

1.2 Solutions

In the mid-1990s, some researchers found an exciting phenomenon that a short single-stranded DNA (ssDNA) ligated to a circular ssDNA template was constantly elongated by some special DNA polymerase (Rubin, 1995 & Liu, 1996). The product of this reaction are long linear ssDNA duplexes with considerable repetitive sequence, which is complementary to the circular ssDNA template. This method of replication referred to as rolling circle amplification (RCA) (Figure 1). Comparing with other conventional methods used for nucleic acid amplification, such as PCR, RCA has its own attractive features. For example, RCA uses a unique isothermal DNA polymerase, which can catalyzes DNA

polymerization at a relatively low and constant temperature. It will reduce the requirement for the instrumentations and no longer need the thermally stable DNA polymerase. Also it does not require a specific primer because any primer complementary to circular template works in RCA. So by using RCA, it can provide thousands-fold amplification in the intensity of the outlet signal and finally improve the detection sensitivity (Cheglakov, 2007),

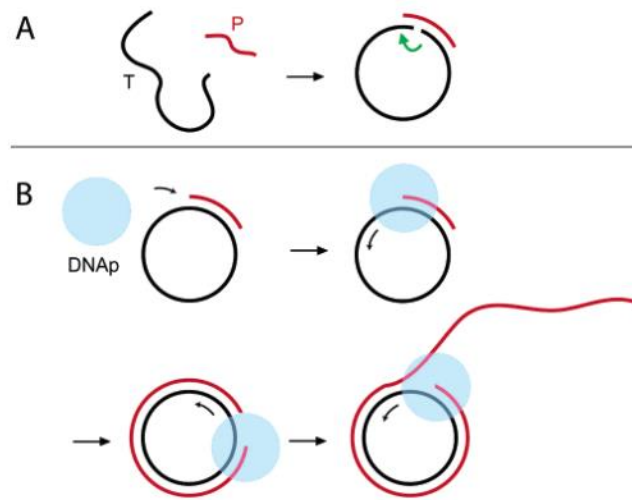
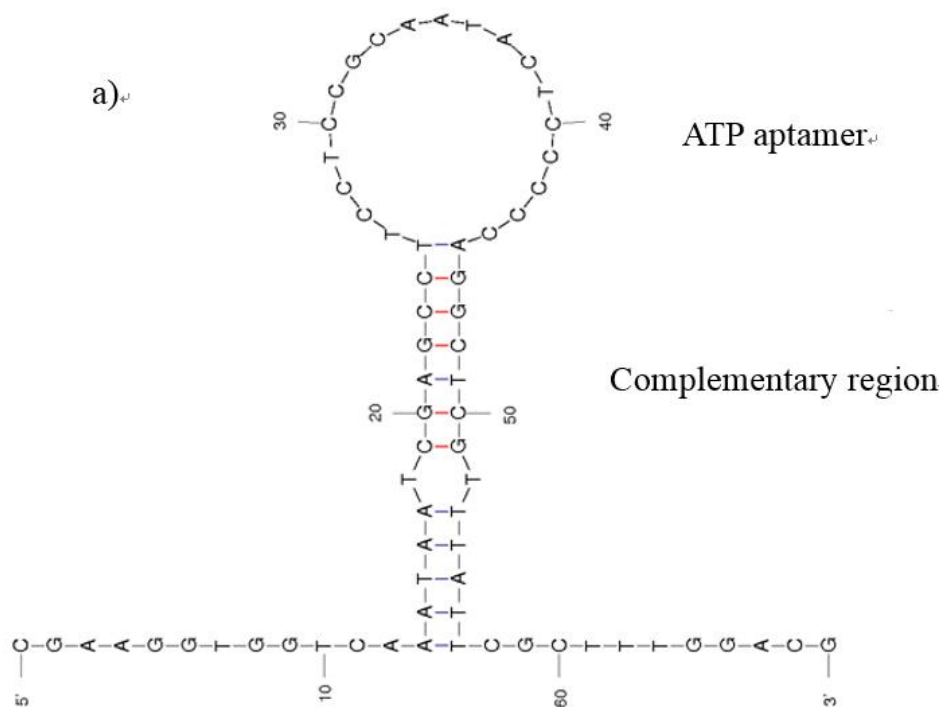


Figure 1. Rolling Circle Amplification, A) after annealing, strand P hybridized strand T. Taq ligase can be used to make a circular DNA strands. B) Strand P as a primer to initialize the amplification and finally with the help of Phi29 DNA polymerase, multiple of linear ssDNA will be produced (Stefan, 2005)

which can exactly solve the problem of low sensitivity in conventional ATP-aptamer-based DNA molecules.

However, even we can make an ATP-binding aptamer-incorporated DNA solution system which can successfully capture and store ATP, the bulk state of solution will be a serious constraint for its further development. So after comparing with other kinds of morphologies, the DNA hydrogel seems to be the best choice. DNA hydrogel has a higher density and greater compressibility than the ones in solution, so it is more convenient for transportation. And in view of its unique feature, DNA hydrogel can create an anisotropic environment in solution, which is the solution unable to do. Last but not least, substrate, such as drug, can diffuse at a unique velocity in liquid environment. This is a point which must be taken into account in pharmaceutical or some other related areas.



hydrogel (Figure 2, a). In addition, I also design another DNA sequence, named T2, which contains a same complementary region as T2-ATP, but it doesn't have an ATP-binding aptamer (Figure 2, b). It plays as a control group in whole project. Takeing all kinds of things into account, our RCA-dependent ATP-binding aptamer-incorporated DNA hydrogel is a really good choice for the ATP capturing.

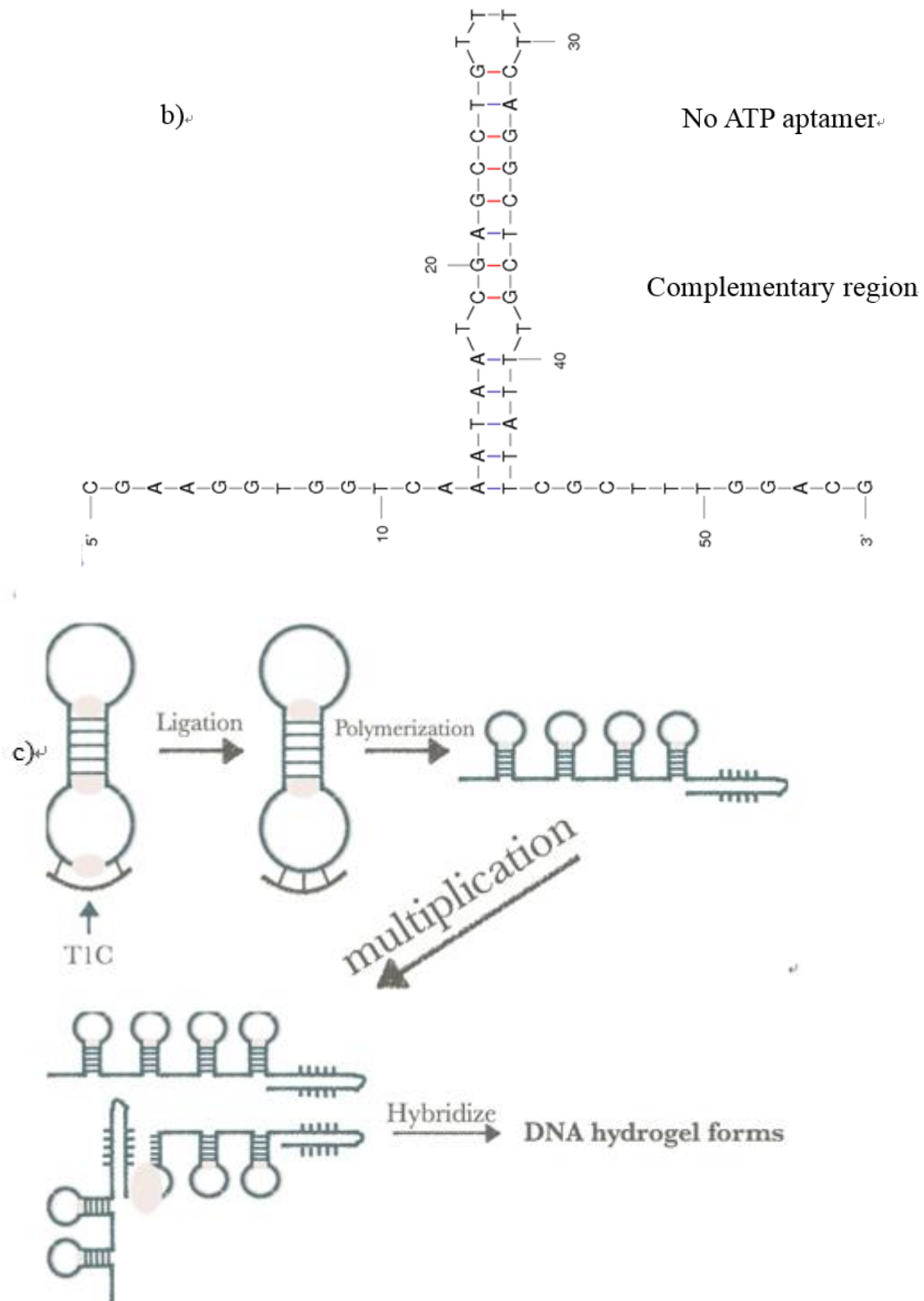


Figure 2, a) Secondary structure of T2-ATP; b) Secondary structure of T2; c)

Schematic diagram of formation of RCA-dependent DNA hydrogel

1.3 Overall Objectives

1. To form a DNA hydrogel by using RCA with specific sequences of linear DNA templates.
2. To determine the ATP capturing capacity of the ATP-binding aptamer-incorporated DNA hydrogel in a laboratory environment.

CHAPRT 2

LITERATURE REVIEW

2.1 ATP aptamer

Nucleic acid aptamers are single-stranded oligonucleotides which can bind to their cognate targets with high affinities and specificities. It has been over 24 years since two groups of researchers (Ellington & Szostak, 1990) used their in-vitro selection and amplification to first successfully isolate certain RNA sequences, which is now called RNA aptamers, from pools of random-sequence libraries (Tuerk, 1990). Later, the DNA-binding aptamer were also found (Ellington, 1992). With further study, researchers have found a broad range of targets of DNA/RNA aptamers from small molecules to proteins, and a lot of aptamers have been selected as therapeutic target for therapeutic interests, like IgE, E2F transcription factor and protein tyrosine kinase 7. Since it appeared, it has been thought to resemble antibody due to its high affinity and specificity. While in some respects, aptamer even seems to be better and more effective than antibody. For example, the flexible dynamics of sequence in aptamer enables it to target virtually all molecules and it can undergo the amplification to improve its sensitivity (Iliuk, 2011). Given all the advantages of aptamers,

they have frequently been used in biosensor (Navani, 2006 & Cho, 2005), detection (Zheng, 2009), bio-imaging (Zhang, 2010) and drug discovery (Ireson, 2006 & Chou, 2005). ATP detection is also one of the important applications and now several sequences and structures of ATP aptamers have been found (figure 2.). This gives investigators a great inspiration and possibility to master ATP.

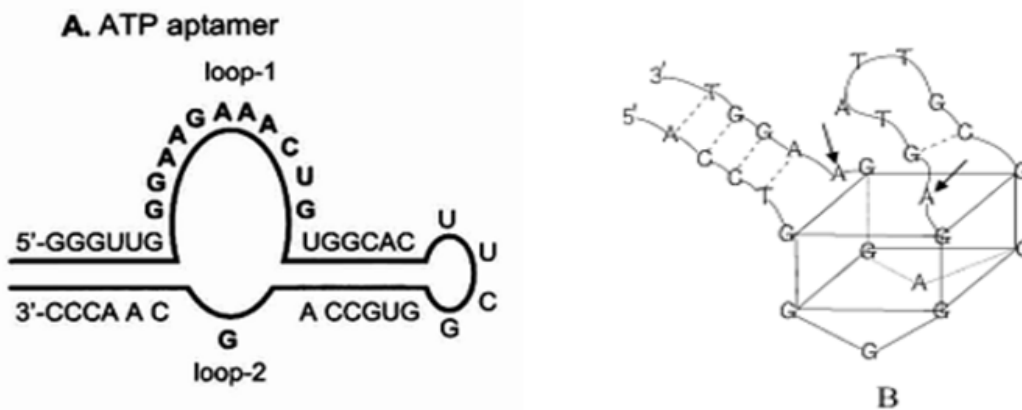


Figure 3. A) ATP aptamer secondary structure (Huang, 2003); B) ATP aptamer tertiary structure (Wang, 2005).

2.2 Rolling Circle Amplification

Rolling circle amplification (RCA) is a high-efficiency nucleic acid replication which can rapidly synthesize multiply copies of ssDNA sequence. Up to 10^5 tandemly repeats of the complementary sequence of circle template will appear in the product sequence. It was first found in the mid-1990s (Rubin, 1995 & Liu, 1996). Comparing with other

conventional methods used for nucleic acid amplification, such as PCR, RCA has its own attractive features. For example, RCA uses a unique isothermal DNA polymerase, which can catalyzes DNA polymerization at a relatively low and constant temperature. It will reduce the requirement for the instrumentations and no longer need the thermally stable DNA polymerase. Also it does not require a specific primer because any primer complementary to circular template works in RCA. RCA can provide thousands-fold amplification in the intensity of the outlet signal and finally improve the detection sensitivity (Cheglakov, 2007). Considering so many advantages over the traditional tool, it is not too hard to understand why it quickly gained the attention for majority of researchers as soon as the technology is invented. And it can also exactly solve the problem of low sensitivity in conventional ATP-aptamer-based DNA molecules, which is invaluable in my project.

CHAPTER 3

3.1 Abstract

Adenosine triphosphate (ATP) is a universal source of energy for all living organisms. It plays a fundamental intracellular and extracellular role in regulation of cellular metabolism, cell signal pathways and biochemical reaction. Its fundamental research and diverging application developed really fast, especially after the discovery of ATP aptamer. With a great development, the ATP aptamer has been applied to a wide range of area, such as biosensor, biomarker and some other detection. In view of the flexibility and versatile sequence and structure of nucleic acid, aptamer technology can be used at almost everywhere and its advantage is also striking, in lots of aspects even better than the antibody. At the same time, rolling circle amplification is in great booming, which can be utilized to rapidly copy multiple of DNA sequence by a circular DNA. Its repeated nucleic acid sequence, provide a really good vector for ATP aptamer in application. In my experiment, we produce a RCA-dependent ATP aptamer-incorporated DNA hydrogel to measure the amount of ATP that can be captured by DNA hydrogel.

3.2 Materials and Methods

3.2.1 RCA-based DNA Hydrogel

The sequence of T2-ATP: 5'-CGAAGGTGGTCAAATAATCGAGCCTTCCTCCGCAA
TACTCCCCCAGGCTCGTTTATTCGCTTTGGACG-3';

The sequence of T2: 5'- CGAAGGTGGTCAAATAATCGAGCCTGTTTTTCAGGCTCG
TTTATTCGCTTTGGACG-3';

The sequence of T1C: 5'- GACCACCTTCGCGTCCAAAGC-3'.

a) Annealing

The original concentrations of T2-ATP, T2 and T1C are 379.5μM, 393.01μM and
354.94μM, respectively. Diluted them to 100μM each by distilled water. Then diluted
them to 2μM solution (50μl scale) each as the following recipe:

- 10×Taq ligase Buffer(New England Biolabs) 5μl
- Sample 1μl
- Distilled Water 44μl

Anneal the 2μM solution from 95°C to 20°C with -1°C per minute by C1000 Touch™
thermal cycler.

b) Ligation

Take the T2-ATP, T2 and T1C out of the thermal cycler. Vortex and slightly centrifuge to
make sure the solution are uniform. Then add the solution as the following recipe:

- T2 or T2-ATP (after annealing) 5μl

- T1C (after annealing) 5μl
- 10×Taq ligase Buffer (New England Biolabs) 1μl
- Distilled Water 8.5μl
- Taq ligase (New England Biolabs) 0.5μl

Before adding the Taq ligase, vortex and slightly centrifuge the tubes to make sure the solution mixes well. No vortex and centrifuge allowed after adding Taq ligase. Otherwise, the Taq ligase will decrease its activity or even denature. Besides, during the whole process, Taq ligase must be kept in ice-bath. After add all solution, put all tubes in 45 °C environment and incubate for 18 hours.

c) Polymerization

Take the T2-ATP and T2 out of the thermal cycler. Vortex and slightly centrifuge. Then add the solution as the following order:

Table 1. DNA hydrogel Preparation for 24 wells in 96-well plate

Type No.	Capture A1-A3	Capture A4-A6	Release A7-A9	Release A10-A12
Gel (50μl)	T2	T2-ATP	T2 growth with ATP	T2 growth with ATP (half conc.)

Type No.	Release B1-B3	Release B4-B6	Release B7-B9	Release B10-B12
Gel (50µl)	T2-ATP growth with ATP	T2-ATP growth with ATP (half conc.)	T2	T2-ATP

Recipe for all above wells:

No. A1-A6 & B7-B12:

- Sample 10.0µl
 - 10×Phi 29 DNA polymerase Buffer (Lucigen™) 4.0µl
 - Distilled Water 28.5µl
 - dNTP 100mM (New England Biolabs) 5.0µl
- (Vortex and slightly centrifuge)
- Inorganic pyrophosphatase (New England Biolabs) 2.0µl
 - Phi 29 DNA polymerase 0.5µl

No. A7-A9 & B1-B3:

- Sample 10.0µl
 - 10×Phi 29 DNA polymerase Buffer (Lucigen™) 4.0µl
 - 1mM ATP 2.5µl
 - Distilled Water 26.0µl
 - dNTP 100mM (New England Biolabs) 5.0µl
- (Vortex and slightly centrifuge)
- Inorganic pyrophosphatase (New England Biolabs) 2.0µl
 - Phi 29 DNA polymerase 0.5µl

No. A10-A12 & B4-B6

- Sample 10.0μl
 - 10×Phi 29 DNA polymerase Buffer (Lucigen™) 4.0μl
 - 1mM ATP 1.25μl
 - Distilled Water 27.25μl
 - dNTP 100mM (New England Biolabs) 5.0μl
- (Vortex and slightly centrifuge)
- Inorganic pyrophosphatase (New England Biolabs) 2.0μl
 - Phi 29 DNA polymerase 0.5μl

After adding all the samples, sealed the 96-well plate by parafilm and place the 96-well plate at 30°C in hybridization oven for 2 days.

3.2.2 ATP measurement

In my project, I used the ATP quantitative kit, which contains Luciferase, to measure the concentration of ATP. Luciferase is a kind of oxidative enzyme that can interact with ATP to produce Luciferyl adenylate, which can keep interacting with O₂ to emit light. The light intensity is positively related to the concentration in solution in theory. So after adding ATP quantitative buffer, put the 96-well plate into plate reader. Then we can get the concentration from the light intensity.

a) ATP standard measurement

Before doing the ATP measurement, we must get the concentration of Luciferase, by which

it is best for the ATP measurement in current concentration. And the result coming from the ATP standard measurement can also be a standard to calculate the concentration of ATP in solution from the light intensity.

From the manufactory, when the concentration of ATP is 1mM, the best dilution rate of Luciferase is around 1/100. So we try the dilution rate, 1/100, 1/200 and 1/300 to do the ATP standard measurement.

Table 2. Distribution of ATP standard measurement

Name	1/100	1/200	1/300	1/100 with half conc of ATP.	1/200 with half conc of ATP.	1/300 with half conc of ATP.	Control
No.	A1, A2	A3, A4	A5, A6	A7, A8	A9, A10	A11, A12	B1, B2
ATP q kit	1/100	1/200	1/300	1/100	1/200	1/300	0
ATP solution	2.5µl ATP/50µl solution	2.5µl ATP/50µl solution	2.5µl ATP/50µl solution	1.25µl ATP/50µl solution	1.25µl ATP/50µl solution	1.25µl ATP/50µl solution	2.5µl ATP/50µl solution

i. Prepare ATP solution (50µl each)

● 2.5µl ATP/50µl solution

- ✧ Distilled Water 42.5µl
- ✧ 10×Phi 29 DNA polymerase Buffer (Lucigen™) 4.0µl
- ✧ 10×Taq ligase Buffer (New England Biolabs) 1.0µl
- ✧ ATP 1mM 2.5µl

● 1.25µl ATP/50µl solution

- ✧ Distilled Water 43.75µl
- ✧ 10×Phi 29 DNA polymerase Buffer (Lucigen™) 4.0µl

✧	10×Taq ligase Buffer (New England Biolabs)	1.0μl
✧	ATP 1mM	1.25μl

Add ATP solution to the 96-well plate first, respectively. Then prepare the additional solution

ii. Add additional solution (50μl each)

- Distilled Water 45μl
- ATP quantitative Buffer 5μl

iii. Add ATP quantitative kit (100μl each)

In view of its high sensitivity, we choose to make the ATP quantitative kit at once and then divide into aliquot.

- 1/100 ATP quantitative kit

✧	Distilled Water	89μl
✧	ATP quantitative Buffer	10μl
✧	ATP quantitative kit	1μl
- 1/200 ATP quantitative kit

✧	1/100 ATP quantitative Buffer	50μl
✧	ATP quantitative Buffer	5μl
✧	Distilled Water	45μl
- 1/300 ATP quantitative kit

✧	1/100 ATP quantitative Buffer	33.3μl
✧	ATP quantitative Buffer	6.7μl
✧	Distilled Water	60μl

After the ATP quantitative kit is made, add them into 96-well plate quickly. The whole

process keep the ATP quantitative kit away from light and avoid the formation in 96-well plate.

b) ATP measurement

From the ATP standard measurement, we get the 1/100 dilution of ATP quantitative kit is best for the ATP measurement in 1mM group. So we use the 1/100 dilution of ATP quantitative kit for this measurement.

i. Prepare ATP solution for standards

● 2.5µl ATP/50µl solution

✧ Distilled Water	42.5µl
✧ 10×Phi 29 DNA polymerase Buffer (Lucigen™)	4.0µl
✧ 10×Taq ligase Buffer (New England Biolabs)	1.0µl
✧ ATP 1mM	2.5µl

● 1.25µl ATP/50µl solution

✧ Distilled Water	43.75µl
✧ 10×Phi 29 DNA polymerase Buffer (Lucigen™)	4.0µl
✧ 10×Taq ligase Buffer (New England Biolabs)	1.0µl
✧ ATP 1mM	1.25µl

● Zero

✧ Distilled Water	45µl
✧ 10×Phi 29 DNA polymerase Buffer (Lucigen™)	4.0µl
✧ 10×Taq ligase Buffer (New England Biolabs)	1.0µl
✧ ATP 1mM	0µl

Put the ATP solution into 96-well plate as following order first,

C1-C3 with 2.5µl ATP/50µl solution, C4-C6 with 1.25µl ATP/50µl solution, C7-C9 with zero.

ii. Prepare post-add solution

- A1-A6 with ATP:
 - ✧ Distilled Water 42.5μl
 - ✧ ATP 1mM 2.5μl
 - ✧ 10×ATP quantitative Buffer 5.0μl

- A7-C9 without ATP:
 - ✧ Distilled Water 45.0μl
 - ✧ 10×ATP quantitative Buffer 5.0μl

iii. Prepare ATP quantitative kit (100μl each)

- 1/100 dilution of ATP quantitative kit
 - ✧ Distilled Water 89μl
 - ✧ ATP quantitative Buffer 10μl
 - ✧ ATP quantitative kit 1μl

Add ATP quantitative kit to each well and do the measurement by plate reader quickly.

The above section is the first trial with 1mM ATP and 1/100 dilution of ATP quantitative kit. Then we also do a second trial with 100μM ATP and 1/10 dilution of ATP quantitative kit.

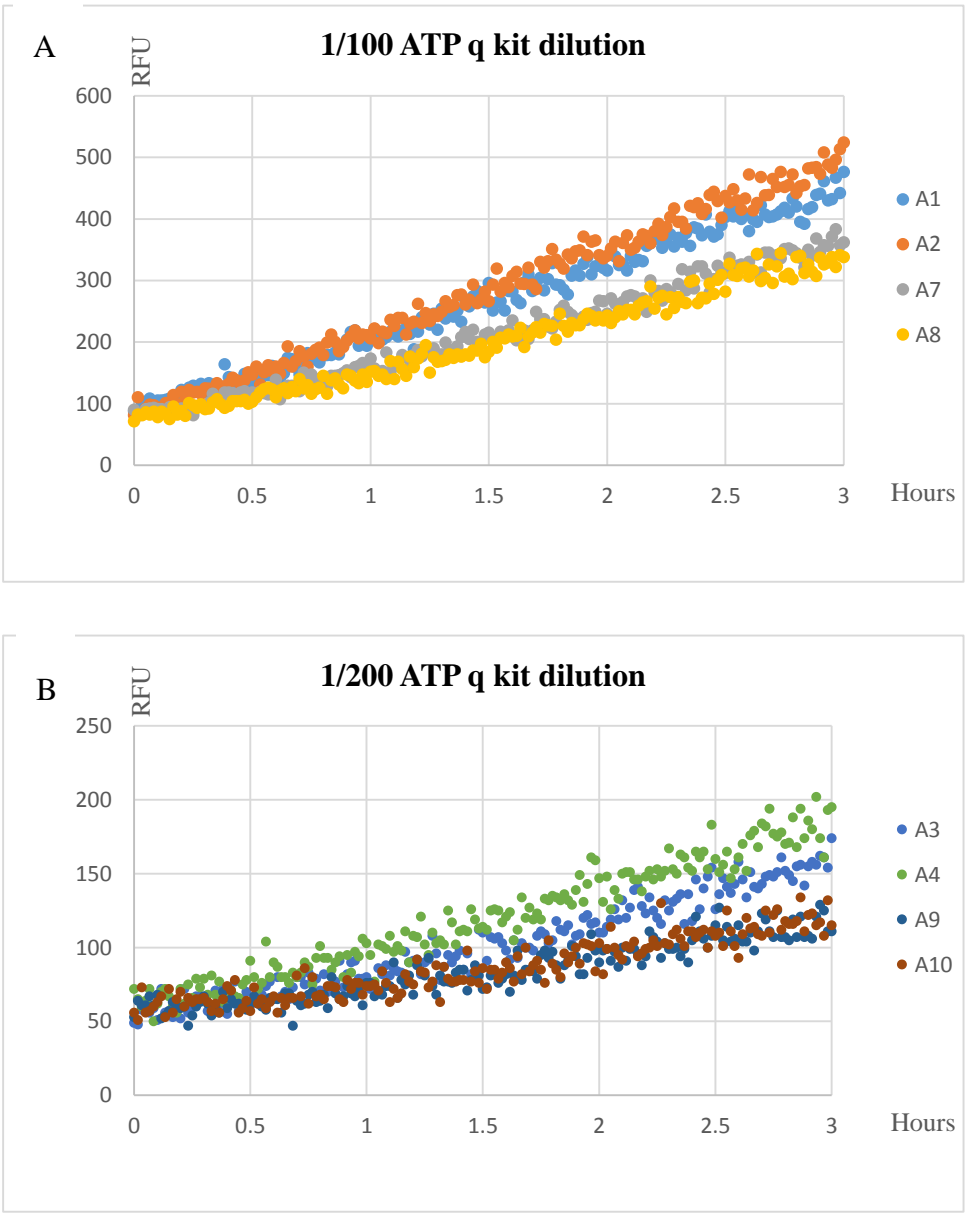
3.3 Results

a) The first trial with 1mM ATP and 1/100 dilution of ATP quantitative kit:

i. Standard measurement

The 1/100 dilution of ATP quantitative kit group has the highest light intensity and the average value of A1 & A2 is almost twice the one in A7 & A8, which use half concentration of ATP. And it has the most obvious difference between the ones in two kinds of concentration. Though the other two groups seem not so bad, but the light intensity is much

smaller which indicates a worse sensitivity.



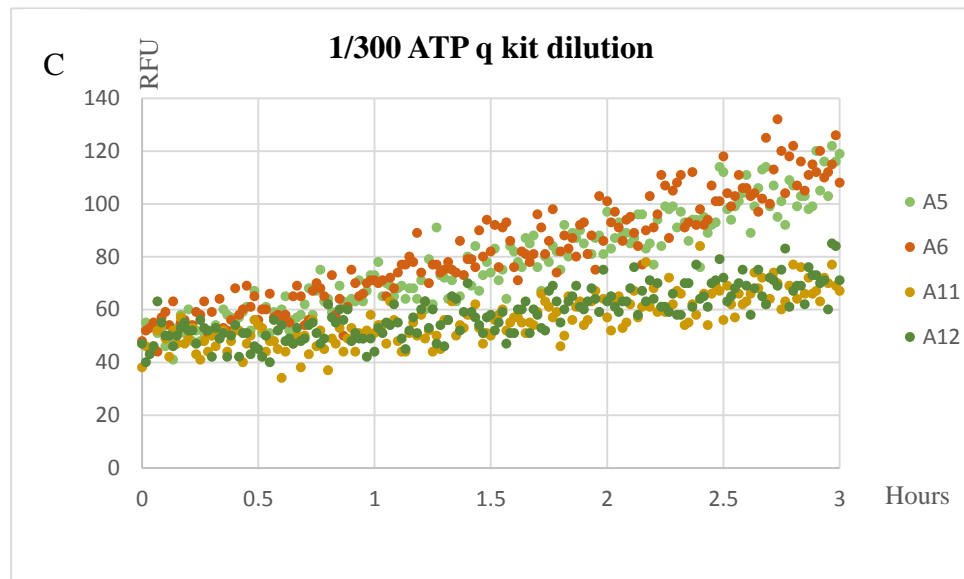
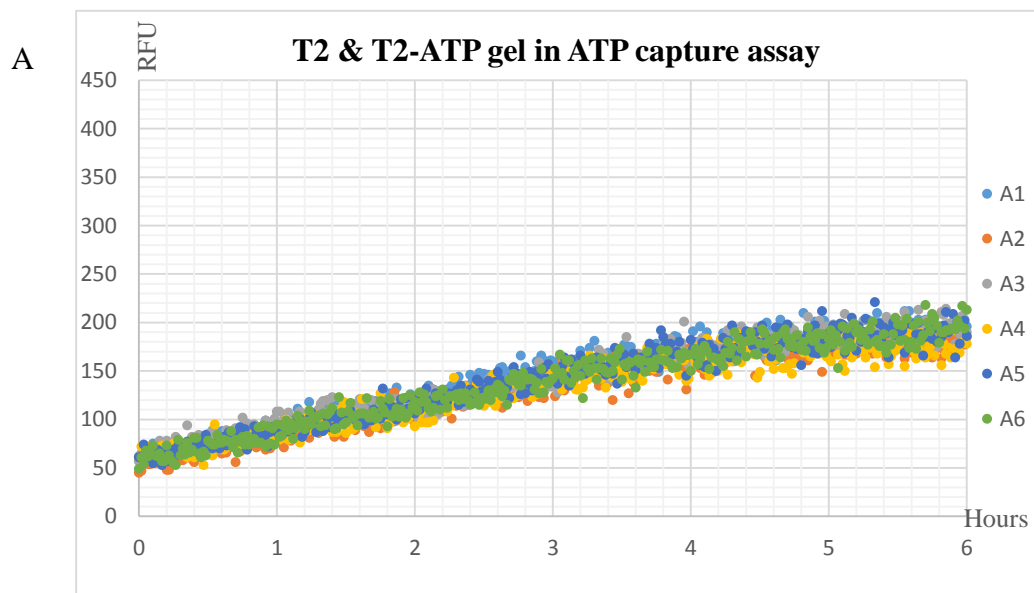
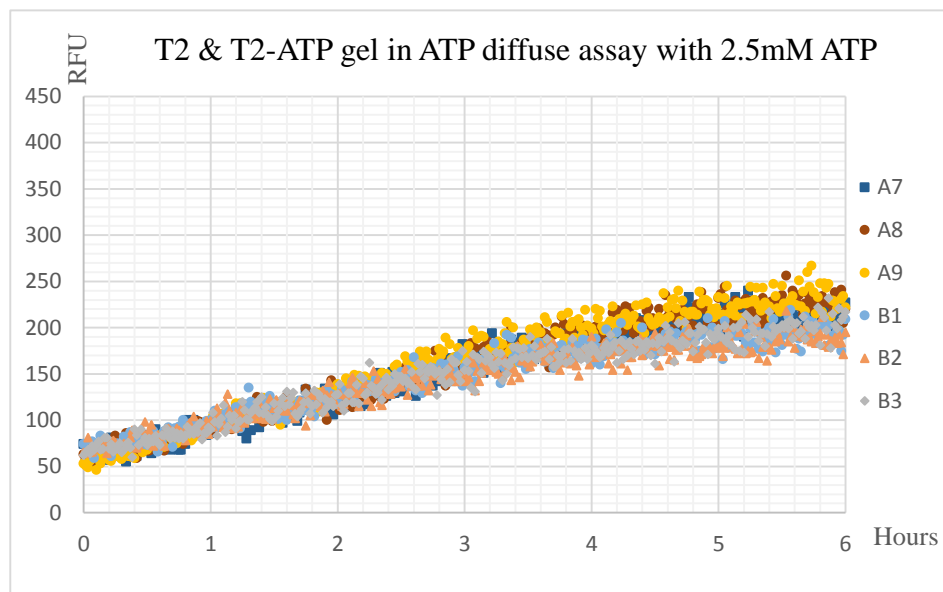


Figure 4. A, B & C show the relationship between light intensity and time in 1/100, 1/200 & 1/300 ATP q kit dilution group

ii. ATP measurement



B



C

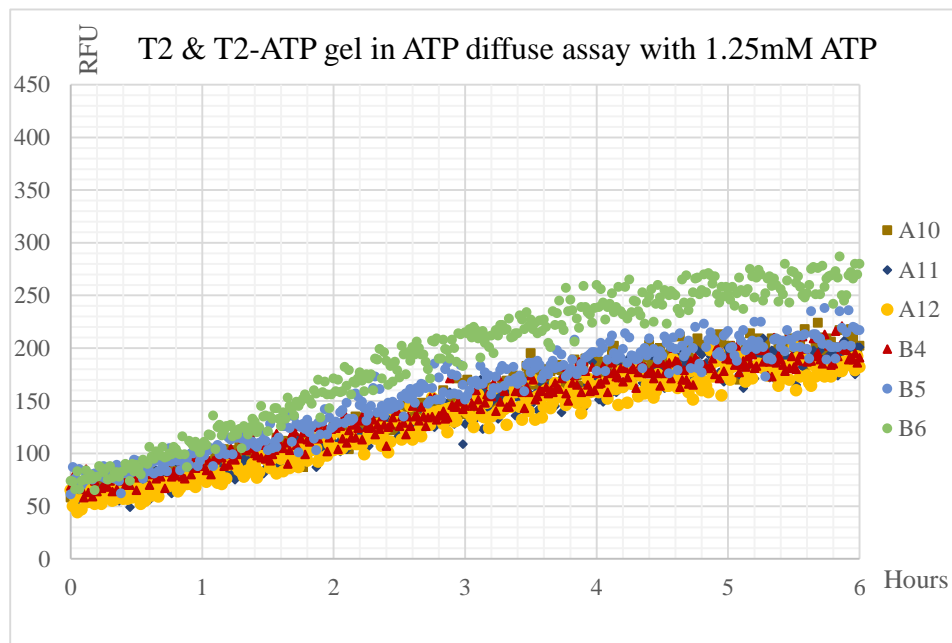


Figure 5. A shows in ATP capture assay, the relationship of concentration of ATP left in the upper solution with time; B & C they show the concentration of ATP diffused to the upper solution after interacting with gel

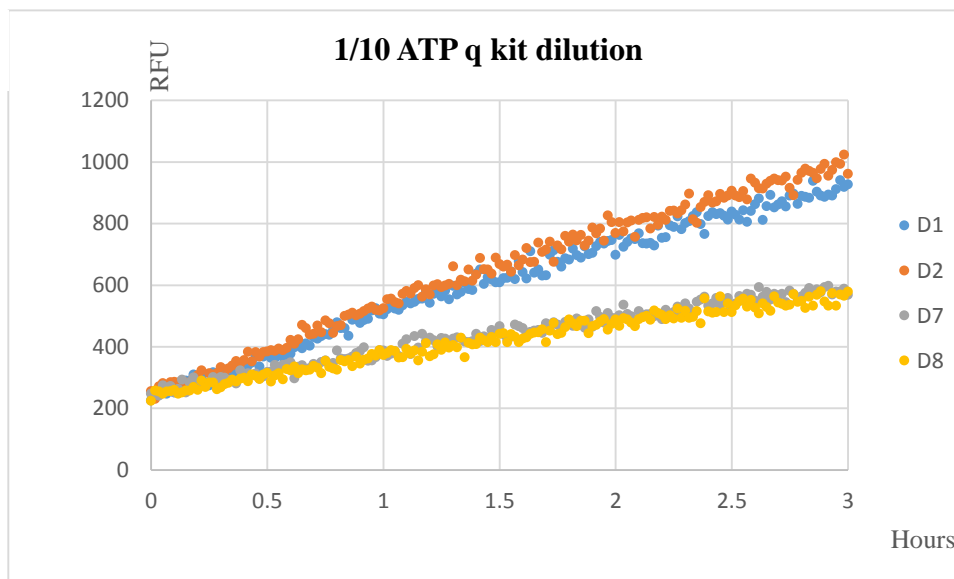
In the T2 & T2-ATP gel in capture assay, the light intensity coming from the interaction of ATP left in solution and Luciferase indicates that the concentrations of ATP in T2 & T2-ATP gel are almost the same. From the ATP release assay, the amount of ATP which diffuse to the upper solution after interaction with DNA hydrogel are still the same between T2 and T2-ATP gel. So from the 1mM ATP assay, we cannot find any evidence to show T2-ATP gel which contained ATP-aptamer can successfully capture ATP molecules.

b) The second trial with 100 μ M ATP and 1/10 dilution of ATP quantitative kit.

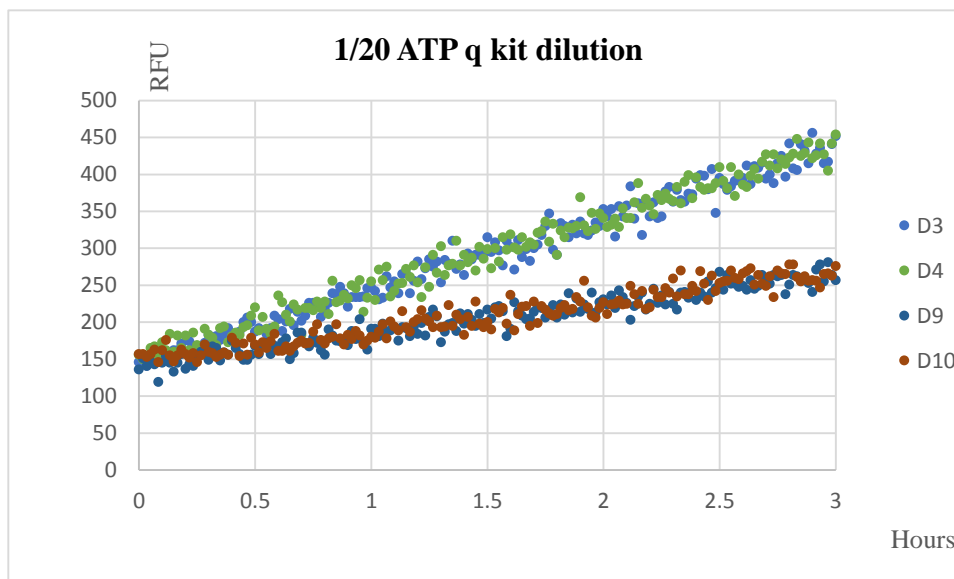
i. Standard measurement

The 1/10 dilution of ATP quantitative kit group has the highest light intensity and the average value of D1 & D2 is almost twice the one in D7& D8, which use half concentration of ATP. Though the value in 1/20 ATP quantitative kit dilution group is also very good, the biggest value is smaller than the one in 1/10 ATP quantitative kit dilution group. So in summary, the one in 1/10 ATP q kit dilution is the best choice for the ATP measurement in 100 μ M group.

A



B



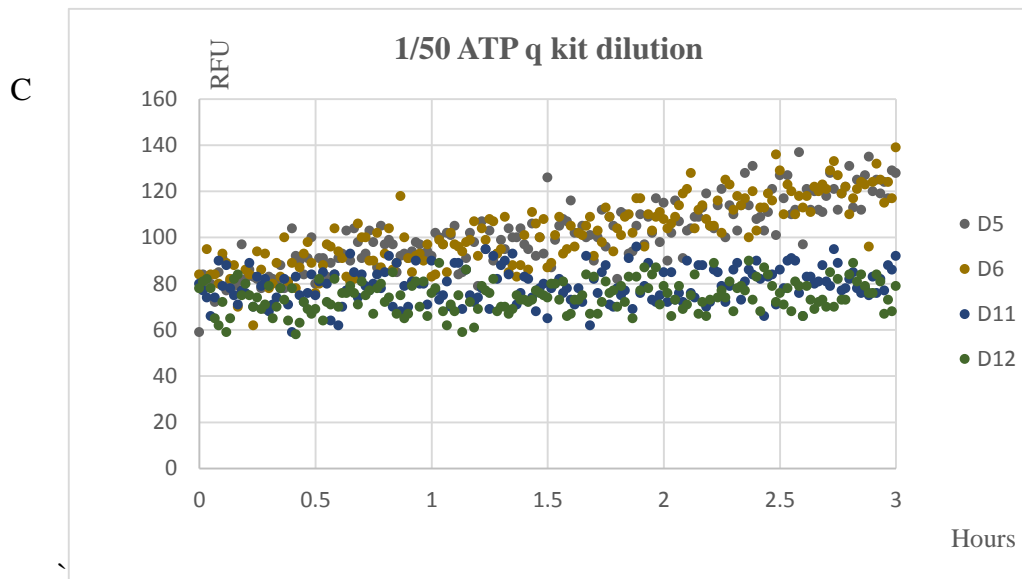
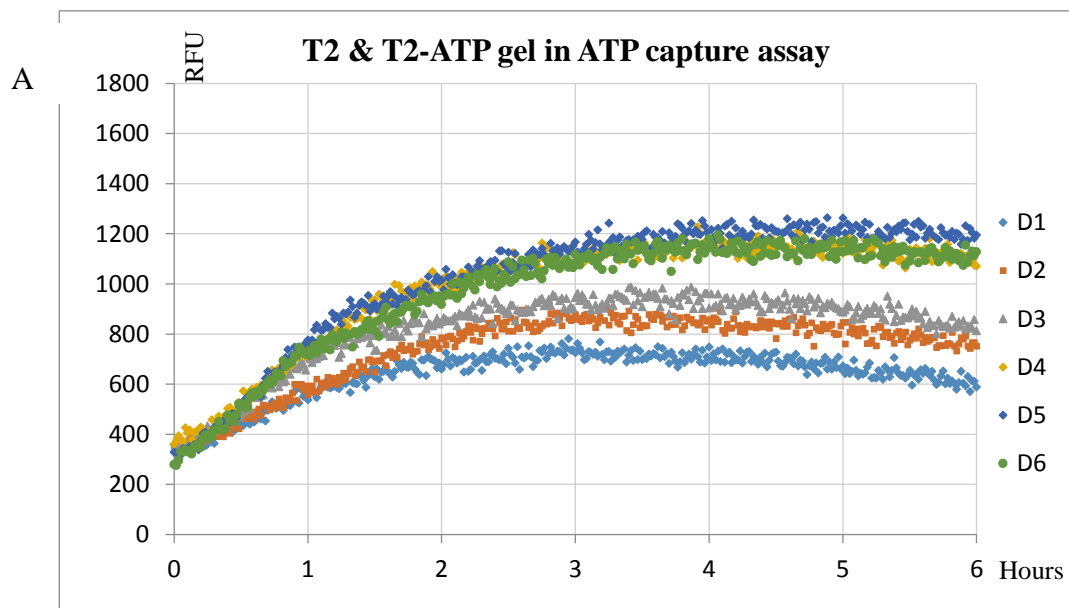


Figure 6. A, B & C show the relationship between light intensity and time in 1/10, 1/20 & 1/50

ATP q kit dilution group

ii. ATP measurement



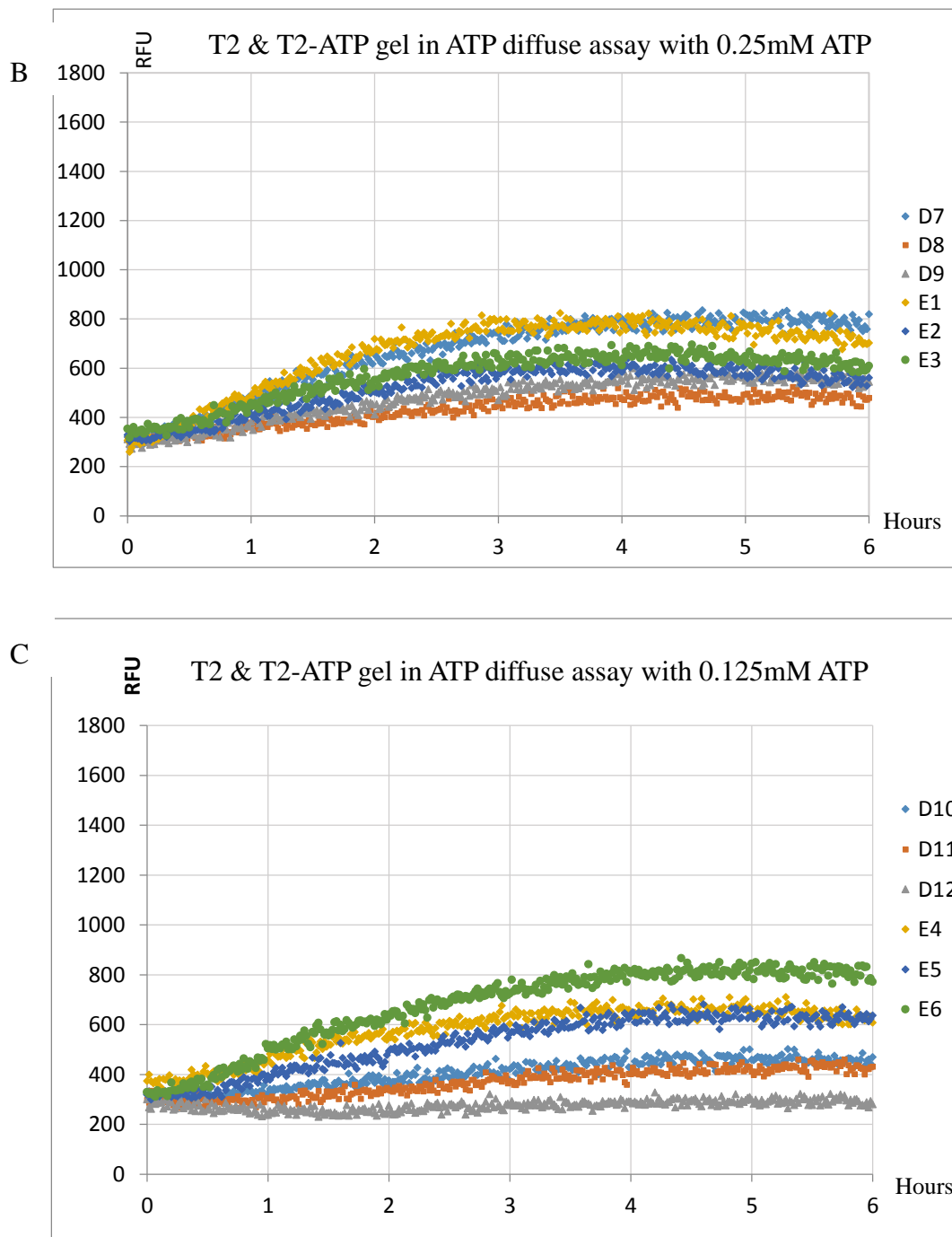


Figure 7. A shows in ATP capture assay, the relationship of concentration of ATP left in the upper solution with time; B & C they show the concentration of ATP diffused to the upper solution after interacting with gel

In the T2 & T2-ATP gel in capture assay in 100mM group, the light intensity coming from the T2 gel solution is weaker than the one in T2-ATP solution, which means there are more ATP left in solution of T2-ATP. It can indicate that T2 gel which doesn't have ATP aptamer can capture or store ATP, while T2-ATP gel can't. From the ATP release assay, the amount of ATP which diffuse to the upper solution after interaction with DNA hydrogel are still the same between T2 and T2-ATP gel. So from the 100 μ M ATP assay, we cannot find any evidence to show T2-ATP gel which contained ATP-aptamer can successfully capture ATP molecules, instead sometime the T2 gel can store or absorb some ATP molecules.

3.4 Discussion

From the results of these two experiments, we cannot find any evidence to support our expected conclusions that our ATP-binding aptamer-incorporated DNA hydrogel can successfully capture ATP. So after reviewing the whole process of this experiment, we find there is one point that has been ignored by us. If the captured ATP can still interact with Luciferase, we can't figure out how much ATP was captured by DNA hydrogel even the fact whether our DNA hydrogel can capture ATP. So in order to solve this problem, we must separate the DNA hydrogel and Luciferase when we are doing measurement. So we

redesigned our current experiment. We form the DNA hydrogel in 1.5ml tube and after adding additional ATP solution and ATP quantitative buffer, shake the tube and ensure that the hydrogel is well-mixed with ATP and other solution. Then we periodically translate certain amount of solution from the tube to another new and clean tube. Add luciferase to the new tube and put it into luminometer to measure the light intensity. Luminometer is a equipment that can provide high performance for bioluminescent and chemiluminescent assay. It has a higher sensitivity than plate reader and it can detect even a very small change of substrate which make the gap like the one in figure 5 possible to be detected. If following this direction, it stands a good chance to get the amount of ATP captured by our hydrogel.

3.5 Conclusion

So far, our ATP-binding aptamer-incorporated DNA hydrogel does not show any obvious capacity of capturing ATP.

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